

Studies on the effect of *Vitex negundo* leaves against the bacteria isolated from marine ornamental fish

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ABSTRACT:

Ornamental fish keeping is growing in leaps and bounds with an ever-increasing number of hobbyists. In the global scene, tropical aquarium fish keeping is considered as the second largest hobby next to photography. Bacterial diseases are the most common infectious problem in ornamental fish rearing which is the set back of this growing industry. Collectively, water quality problems exceed bacterial diseases in the area of pet fish morbidity and mortality. The present attempt is a trail for control the diseases and possible cure with the leaves extract of *Vitex negundo*. Infected *Dascyllus trimaculatus* fishes were collected from the marine aquarium of Annamalai University and the bacteria such as *Vibrio tarachuri*, *V. harveyi*, *Enterobacter agglomerans*, *Aeromonas hydrophila* and *Pseudomonas fluorescences*, *Protease* sp., *Citrobacter* sp., *Flavobacterium* sp., were isolated from the fishes and identified. The *V. negundo* leaves were extracted with different solvents like methanol, acetone and aqueous. The results revealed that the methanol extract produced minimal inhibitory concentration at 300 µg/ml against *Protease* sp. and acetone extract showed good aminimal inhibitory concentration against *Protease* at 400 µg/ml. Methanol extract of *V. negundo* leaves shows the presence of alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, phenolic compounds, proteins, free amino acids, flavonoids, lignin and volatile. Based on the results it is concluded that the *V. negundo* leaves had significant effect to control the bacteria isolated from one of the important damsel fish, *D. trimaculatus* which plays major role in the aquarium trade.

Key words: Bacteria, crude extract, *Vitex negundo* leaves, minimum inhibitory concentration, zone of inhibition.

INTRODUCTION

The fantastic shapes, brilliant colours and fascinating patterns of marine ornamental fishes have won the hearts of millions of people, and hence they are aptly called “living jewels” [1]. The marine ornamental fishes have tremendous variations in colour pattern. The aquarists and scientists are puzzled by the different colour patterns that may occur in a single species. The ornamental animals are of the highest value, that can be harvested from the reef system and which supports over 4000 species of fish, 800 species of reef building corals and a great number of other invertebrates and sponges. The important marine ornamental fishes in the Indian waters are clowns, angels, damsels, squirrels, surgeons, butterflies, triggerfish, moorish idol, wrasses, etc.

Among the marine ornamental fishes, damsels are very popular among aquarists due to their small size, bright colors, quick acclimatization captivity and interesting display behavior [2]. The most common marine aquarium fish, the three spot Damsel fish, *Dascyllus trimaculatus* playing a major role among the aquarists [3]. *D. trimaculatus* has grey to black body with two lateral white spots and one between the eyes & it grows up to 14 cm in length. Coloration is somewhat variable, the spot on the forehead may be absent and the lateral spot very much reduced [4]. It feeds on algae, copepods and other planktonic crustaceans.

Diseases are found to crop up which can badly affect the profitability of the ventures. Bacterial disease is the most common infectious problem of ornamental fishes, and most of the bacterial infections are caused by gram-negative organisms [5]. Antibiotics merely control the growth of bacteria in a fish long enough for its immune system to eliminate them. But this therapy

fails because of the development of resistant character developed by microorganisms and it creates side effects to the host. Due to this, much attention has paid to herbal or traditional medicines [6], [7], [8], and [9].

Vitex negundo Linn (Verbenaceae), a large aromatic shrub found throughout the greater part of India at warmer zones and ascending to an altitude of 1500 m in outer, Western Himalayas. It has been claimed to possess many medicinal properties [10]. The Sanskrit word “nirgundi” literally means that which protects the body from diseases. Though almost all of its parts are used in Ayurvedic and Unani systems of medicine, the leaf and root extracts are the most important in the field of medicine and drug [11], [12] & [13]. The leaves contain fragrant, volatile oil and resins [14] and the fruits contain resins, astringent organic acids, alkaloids and a pigment. It was also reported that, the plant also contains alkaloids, glycosides, flavonoids, reducing sugars, sterols, resin and tannins [15]. In the present study bacteria were isolated from infected damsel fish *D. trimaculatus* and screened the antibacterial activity of *V. negundo* leaves and also studied the phytochemical parameters of the *V. negundo*.

MATERIALS AND METHODS

Plant material and extraction

Fresh leaves of *V. negundo* were collected from in and around Trichy district, Tamil Nadu, India. After shade drying, the leaves were powdered by using mechanical grinder [16]. Five grams of the dry powder were extracted in 50 ml of methanol, acetone and aqueous solutions using rotary shaker for 30 min at 100 rpm and the extract was separated by centrifugation at 10,000 rpm for 30 min. After centrifugation the solvent portion was collected and evaporated.

Bacterial strains

Bacteria were isolated from moribund stages of the fish, *D. trimaculatus*. Infected parts of the fishes were collected and serially diluted and plated on Zobell marine agar and nutrient agar with 50% seawater, incubated at 37⁰ C for 24 hr. After incubation, colonies were pure cultured and subcultured on Zobell marine agar slants [17]. The morphological and biochemical tests were performed according to Bergey's manual of systematic Bacteriology [18].

Disc diffusion method

Antibacterial activity of *V. negundo* leaves was tested against the bacteria isolated from infected damselfish *D. trimaculatus* by disc diffusion method [19] & [20]. The antibacterial susceptibility tests were performed on Muller–Hinton Agar medium. Twenty milliliter of Agar medium were poured into the plates to obtain uniform depth and allowed to solidify. The standard inoculum suspension (106 c.f.u./ml) were streaked over the surface of the media using sterile cotton swab to ensure the confluent growth of the organism. The 5 mm diameter discs were prepared with Whatman No.1 paper. About 500µg of crude extract was impregnated on the filter paper discs. The discs were placed on the surface of the plate with sterile forceps and pressed gently to ensure contact with the inoculated agar surface. Oxytetracyclin (500 µg/disc) was used as positive reference standard to determine the sensitivity of the tested strains and 5% DMSO was used as negative control. Finally, the inoculated plates were incubated at 37⁰C for 24 hrs and the inhibition zones were observed including the diameter of the disc (5 mm). All the experiments were carried out in triplicate.

Determination of minimum inhibitory concentration

Minimum inhibition concentration (MIC) of methanol, acetone and aqueous extracts of *V. negundo* leaves was determined by broth dilution assay method [21] & [22]. The inoculation of the bacterial strains were prepared from 12-hr old broth cultures and suspensions were adjusted to standard turbidity (106 c.f.u./ml). *V. negundo* leaves extract was dissolved in 5% DMSO to obtain 2,000 µg /ml stock solution. About 0.5 ml of stock solution was incorporated into 0.5 ml of Muller–Hinton broth to give concentrations of 1000, 500, 250, 125, 62.5, and 31.25 µg/ml. The stock concentration was changed again and again for analysis of MIC value. Fifty microliter of standard suspension of the test organism was transferred onto each test tube. The control tube contained only organism and devoid of *V. negundo* leaves extract and 5% DMSO was used as blind control. The culture tubes were incubated at 37⁰ C for 24 h.

Qualitative analysis of phytochemical compounds

Alkaloids, carbohydrates, glycosides, phytosterol, oils, fats, saponins, tannins, phenolic compounds, proteins,

free amino acids, gums, mucilages, flavonoids, lignin and volatile oils were done by adapting the method proposed by [23].

RESULTS AND DISCUSSION

Based on the present investigation totally 8 bacteria were isolated and identified as *Vibrio tarachuri*, *V. harveyi*, *Enterobacter agglomeranus*, *Aeromonas hydrophila*, *Pseudomonas fluorescences*, *Protease* sp., *Citrobacter* sp., and *Flavobacterium* sp., (Table -1). Aqueous, methanol and acetone extracts were subjected for the study and it revealed that both methanol and acetone extracts shown good activity at 500 µg/disc concentration. Methanol extract exhibited good activity against *Protease* sp. (28 mm), *V. harveyi* (22 mm), *P. fluorescences* (22 mm), *V. tarachuri* (20 mm), *Flavobacterium* sp. (18 mm) and *Aeromonas hydrophila* (16 mm). Acetone extract shown fine activity against *Protease* sp. (26 mm), *Vibrio harveyi* (18 mm), *Aeromonas hydrophila* (18 mm) and aqueous extracts exhibited minimum activity against all the pathogens (Fig. 1).

Minimal inhibitory concentration of *V. negundo* leaves was tested against 8 types of isolated strains. Methanol extract produced minimal inhibitory concentration at 300 µg/ml against *Protease* sp. and acetone extract showed good minimal inhibitory concentration against *Protease* at 400 µg/ml (Fig. 2). Principal component of bioactivity in plants are phytochemical constituents; they induced precipitation of protein, induce immuno modulation, etc. Methanol extract of *V. negundo* leaves shows the presence of alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins & phenolic compounds, proteins and free amino acids, flavonoids, lignin, volatile (Table. 2).

Bacterial diseases are the most common infectious problem of ornamental fishes. In the present study, the gram negative bacteria were isolated and the results are lined in the report of [24] Gram-negative organisms causes majority of bacterial infections including the following pathogenic genera: *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium* (*flexibacter*), *Mycobacterium*, *Pseudomonas*, and *Vibrio* (Fig. 1).

V. negundo is found throughout India. The leaf extracts and roots are the most important product in the field of medicine and drug [13]. The methanol, acetone and aqueous extract of *V. negundo* leaves studied against the bacteria such as *V. tarachuri*, *V. harveyi*, *Protease* sp., *Citrobacter* sp., *Flavobacterium* sp., *Enterobacter agglomeranus*, *A. hydrophila*, and *P. fluorescences* isolated from the infected region of damselfish, *D. trimaculatus*. The results obtained from the present study are in lined with various scientific reports given by [12], [25] & [15]. According to [26] *V. negundo* leave extract was potential against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and

Pseudomonas aeruginosa. Minimal inhibitory concentration of *Vitex negundo* was found to be between 200 - 1200 µg/ml and this was also similar to the findings of [15].

Antibacterial activity may be due to phytochemical content of the plant [27] and [28] reported that *Vitex negundo* have many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids. The phytochemical investigation of methanolic extract of *V. negundo* leaves resulted in the isolation of eight compounds under silicagel VLC, CC and preparative TLC. In the present study also the presence of alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, phenolic compounds, proteins, free amino acids, flavonoids, lignin and volatile were observed (Table 2) and which have antipyretic, anti-arthritis and anti-inflammatory properties and is used for human diseases also [29] and [30].

A number of studies have been conducted for control and cure the human diseases with the help of *V. negundo* plant. But based on the literature survey, treating the marine ornamental fish using this plant is empty. This is a trail investigation made for the possible remedies against bacterial diseases in marine ornamental fishes, especially in damselfish, *Dascyllus trimaculatus* which is the permissive out put to the growing industry. This preliminary *in vitro* screening indicated good results and the detailed study in this line is requested.

ACKNOWLEDGMENTS

Authors are thankful to the authorities of the Annamalai University for providing facilities and the University Grants Commission (UGC), New Delhi for financial support.

Table: 1. BIOCHEMICAL TEST

Tests	I	II	III	IV	V	VI	VII	VIII
Gram staining	-	-	-	-	-	-	-	-
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Motility	M	M	M	M	M	M	M	M
Indole	+	+	+	-	-	-	+	-
MR	+	-	+	+	-	+	+	+
VP	-	-	-	-	-	+	-	-
Citrate	-	+	-	+	+	+	-	+
Urease	-	+	+	+	+	-	-	-
Oxidase	-	-	-	-	+	-	+	-
Catalase	-	+	+	+	-	-	+	-
TSI	A/A	A/K	A/K	A/A	K/A	K/A	K/A	A/A
G ⁺	-	-	-	-	-	-	-	-
H ₂ S	-	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+
Lactose	+	-	+	+	+	+	+	+
Mannitol	+	-	-	+	+	+	+	+
Sucrose	+	+	-	+	+	+	-	+

M = Motile, NM = Non motile, + = Positive, - = Negative

- I - *Vibrio tarachuri*
- II - *Vibrio harveyi*
- III - *Protease* sp.
- IV - *Citrobacter* sp.
- V - *Flavobacterium* sp.
- VI - *Enterobacter agglomeranus*
- VII - *Aeromonas hydrophila*
- VIII - *Pseudomonas fluorescences*

Table 2. PRELIMINARY PHYTOCHEMICAL SCREENING

S.No	Phytoconstituents	Methanol
1	Alkaloids	+
2	Carbohydrates	+
3	Glycosides	+
4	Phytosterols	+
5	Saponins	+
6	Fixed oils & Fats	-
7	Tanins & Phenolic compounds	+
8	Proteins & free amino acids	+
9	Gums & Mucilage	-
10	Flavonoids	+
11	Lignin	+
12	Volatile	+

(+) = Presence (-) = Negative

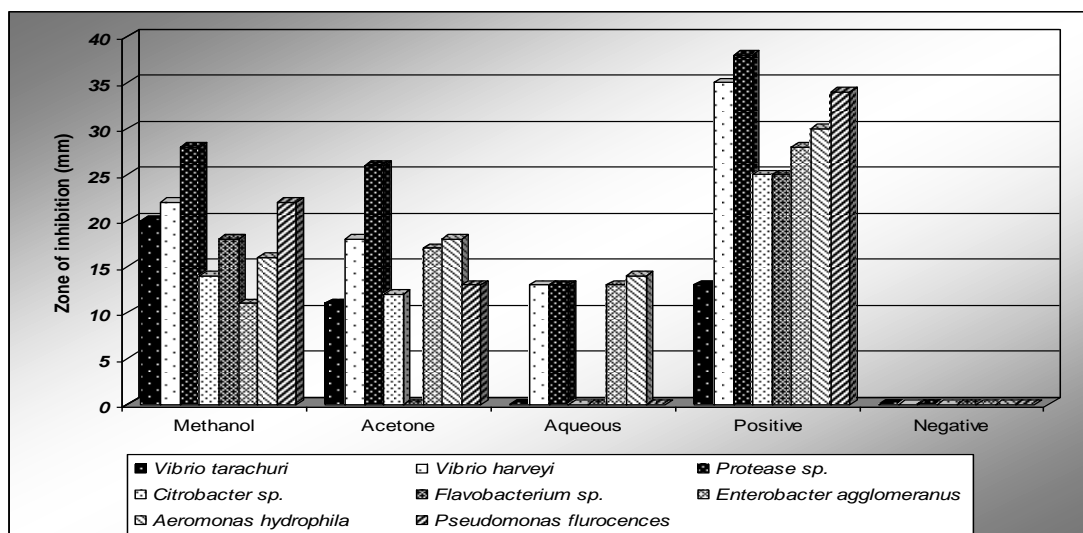


Figure 1. Antibacterial activity of *Vitex negundo* leaves

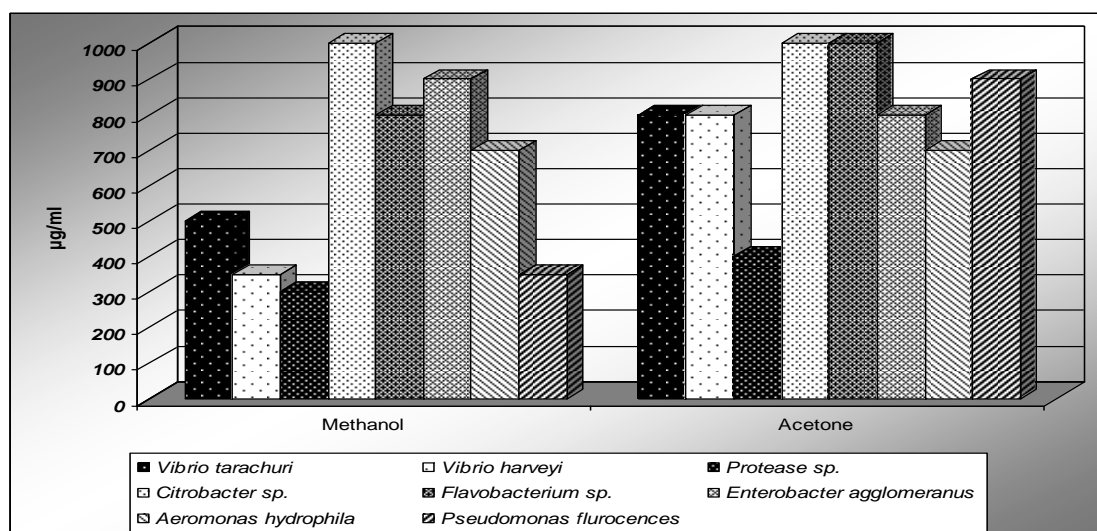


Figure 2. Minimal inhibitory concentration of *Vitex negundo* leaves

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